Antepartal insulin-like growth factor 1 and insulin-like growth factor binding protein 2 concentrations are indicative of ketosis in dairy cows

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Abstract: A study involving a small number of cows found that the concentrations of insulin-like growth hormone 1 (IGF1) may be a useful predictor of metabolic disease. Further, IGF1 may provide also a pathophysiological link to metabolic diseases such as ketosis. The objective of the current study was to test whether the low antepartal total IGF1 or IGF1 binding protein (IGFBP) concentrations might predict ketosis under field conditions. Clinical examinations and blood sampling were performed antepartum (262-270 d after artificial insemination) on 377 pluriparous pregnant Holstein Friesian cows. The presence of postpartum diseases were recorded (ketosis, fatty liver, displacement of the abomasum, hypocalcemia, mastitis, retention of fetal membranes, and clinical metritis or endometritis), and the concentrations of IGF1, IGFBP2, IGFBP3, and nonesterified fatty acids were measured. Cows with postpartum clinical ketosis had lower IGF1 concentrations antepartum than healthy cows. The sensitivity of antepartal IGF1 as a marker for postpartum ketosis was 0.87, and the specificity was 0.43; a positive predictive value of 0.91 and a negative predictive value of 0.35 were calculated. The cows with ketosis and retained fetal membranes had lower IGFBP2 concentrations compared with the healthy cows. It can be speculated that lower IGF1 production in the liver during late pregnancy may increase growth hormone secretions and lipolysis, thereby increasing the risk of ketosis. Lower IGFBP2 concentrations may reflect the suppression of IGFBP2 levels through higher growth hormone secretion. In conclusion, compared with nonesterified fatty acids as a predictive parameter, IGF1 and IGFBP2 may represent earlier biomarkers of inadequate metabolic adaptation to the high energy demand required postpartum.

DOI: https://doi.org/10.3168/jds.2014-8885

Originally published at:
Piechotta, M; Mysegades, W; Ligges, U; Lilienthal, J; Hoeflich, A; Miyamoto, A; Bollwein, Heiner (2015). Antepartal insulin-like growth factor 1 and insulin-like growth factor binding protein 2 concentrations are indicative of ketosis in dairy cows. Journal of Dairy Science, 98(5):3100-3109.
DOI: https://doi.org/10.3168/jds.2014-8885

PRE-PARTUM IGF1 LEVEL PREDICT KETOSIS

Piechotta

The uncoupling of the somatotropic axis is one of the key metabolic adaption pathways during the transition from late pregnancy to early lactation and is reflected by increasing growth hormone concentrations and a decreasing hepatic production of IGF1. This study shows that low total IGF1 concentrations in clinically healthy pluriparous Holstein Friesian cows in late pregnancy (262 - 270 d after artificial insemination) predicted the risk of clinical ketosis after calving. The sensitivity was 51 % and the specificity 83 % (threshold IGF1 = 62 ng / mL, and IGF1 may represent an early biomarker of inadequate metabolic adaptation to the high-energy demand required post partum.

Antepartal IGF1 and IGFBP2 concentrations are indicative of ketosis in dairy cows

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ABSTRACT

A study involving a small number of cows found that the concentrations of insulin-like growth hormone-1 (IGF1) may be a useful predictor of metabolic disease. Further, IGF1 may provide also a pathophysiological link to metabolic diseases such as ketosis. The objective of the current study was to test whether the low antepartal total IGF1 or IGF1 binding protein (IGFBP) concentrations might predict ketosis under field conditions. Clinical examinations and blood sampling were performed ante partum (262-270 d after artificial insemination (AI)) on 377 pluriparous pregnant Holstein Friesian cows. The presence of postpartum diseases were recorded (ketosis, fatty liver, displacement of the abomasum, hypocalcemia, mastitis, retention of fetal membranes, and clinical metritis/endometritis), and the concentrations of IGF1, IGFBP2, IGFBP3 and non-esterified fatty acids were measured. Cows with post partum clinical ketosis had lower IGF1 concentrations ante partum than healthy cows. The sensitivity of antepartal IGF1 as a marker for post partum ketosis was 0.87, and the specificity was 0.43; a positive predictive value of 0.91 and a negative predictive value of 0.35 were calculated. The cows with ketosis and retained fetal membranes had lower IGFBP2 concentrations compared with the healthy cows. It can be speculated that lower IGF1 production in the liver during late pregnancy may increase growth hormone secretions and lipolysis, thereby increasing the risk of ketosis. Lower IGFBP2 concentrations may reflect the suppression of IGFBP2 levels through higher growth hormone secretion. In conclusion, compared with non-esterified fatty acids as a predictive parameter, IGF1 and IGFBP2 may represent earlier biomarkers of inadequate metabolic adaptation to the high-energy demand required post partum.

Keywords: dairy cow, transition period, IGF1, IGFBP2, ketosis

INTRODUCTION

The growth hormone (GH)-IGF axis is an important endocrine control center for metabolic adaptation in dairy cows. Studies have indicated that IGF1 may be useful as a predictive marker for postpartum (pp) production diseases (Piechotta et al., 2012) or for successful early ovulation pp (Kawashima et al., 2007). Moreover, from in-vitro studies, IGF1 is known to be an important signal for gluconeogenesis (Wang et al., 2012); therefore, a physiological association between IGF1 and particularly metabolic diseases, such as ketosis, is likely, but the exact mechanisms between the somatotropic axis and the pathogenesis of ketosis are not well studied. Studies have attempted to influence the GH-IGF1 axis by administering bovine somatotropin (bST) antepartum (ap). Although ap bST administration did not affect the incidence of hyperketonemia, dry matter intake or clinical ketosis (Gohary et al., 2014), a clear association between low IGF1 concentrations and metabolic production diseases was evident (Piechotta et al., 2012). Cows that were classified based on low versus high IGF1 levels revealed that there were no differences in the hepatic growth hormone receptor mRNA expression (Piechotta et al., 2013, 2014), which might explain why the bST administration did not have an effect on the incidence of ketosis. However, from these studies, it was not clear which factors might be responsible for the association between low total IGF1 levels and the incidence of metabolic diseases. Moreover, the number of cattle used in the study was low, and only IGF1 was measured (Piechotta et al., 2012). However, IGF1 is bound to six different high affinity IGF binding proteins (IGFBP) that determine the half-life of IGF1 and its delivery through the endothelium to the target cells. The IGFBP concentration might be one factor for different total IGF1 concentrations. Therefore, the concentrations of the two most abundant IGFBPs (IGFBP2 and IGFBP3) were determined in the present study with a greater

number of animals under field conditions to clarify whether ap total IGF1 or IGFBP concentrations might predict the risk of ketosis or other pp production diseases.

MATERIALS AND METHODS

Animals

In one large-scale dairy farm (~ 1300 cows) in eastern Germany (Göritz, Brandenburg), 377 pluriparous Holstein Friesian cows in late pregnancy (2^{nd} to 4^{th} lactation, 305-day milk yield of 11,200 ± 97 kg [mean ± SEM]) were examined, and blood samples were obtained. The experimental procedure was approved by the German legislation responsible for animal welfare (Landesamt für Umwelt, Gesundheit und Verbraucherschutz, Abteilung Verbraucherschutz in Frankfurt (Oder); 23-2347-A19-3-2010). The cows were housed during all seasons in a free-stall barn with rubber mats and were fed automatically by a band-conveyor system twice daily with a total mixed ration depending on the lactation period (Table 1, 2). The cows were provided with a mineral supply (Deutsche Vilomix Tierernährung GmbH, Neuenkirchen-Vörden, Germany), and they had free access to water. The cows were kept in groups depending on the lactation interval (early [50 days pp], mid, late, and dried-off). The cows were dried-off approximately six weeks before the expected calving date. Approximately ten days before calving, the cows were placed in a free-stall barn with straw bedding in which the cows were monitored for signs of birth every two hours by the farm staff. The cows were milked three times daily, and the milk yields were recorded once a month by the routine control office (Landeskontrollverband Brandenburg, Brandenburg, Germany).

Monitoring of Health Status and Blood Sampling

The cows were monitored daily by the farm staff via observing the feed intake ap and pp and recording milk yield, milk character and the udder after calving. If the cows showed either a

115 reduction in feed intake and/or milk yield, a farm veterinarian was summoned, and the cows
116 were diagnosed and treated. Moreover, the cows were examined clinically by a study
117 veterinarian once ap between 262-270 d after artificial insemination (AI) and two times pp at
118 three (+ 3 wk: 16 – 21 d pp) and four weeks (+ 4 wk: 22 – 28 d pp) after calving. At each
119 examination, behavior, posture, body temperature and BCS were recorded (Edmonson et al.,
120 1989). Additionally, the milk yield during the previous lactation was documented. A
121 gynecological examination was performed to assess the occurrence of metritis/endometritis in
122 accordance with Sheldon et al. (2008). After each examination, a blood sample was obtained
123 from a coccygeal vessel directly into tubes with EDTA and without any anticoagulants to
124 acquire serum (Sarstedt, Nümbrecht, Germany). The EDTA containing the samples was kept
125 on ice, whereas the serum samples were kept until clotting at ~ 20 ° C. After centrifugation
126 (2000 g, 15 min, 4 ° C, Hettich EBA 20, Germany), which occurred within two hours after
127 sampling, the samples were kept at – 20 ° C until further analyses. Moreover, if a decrease in
128 the milk yield or feed intake of the cows was detected by the farm stuff, the farm veterinarian
129 conducted a clinical examination and recorded the diagnosis according to the stated
130 definitions provided below. The day of AI and the calving date were recorded using the farm
131 management software (“HERDE2”, dsp-agrosoft, Paretz, Germany). A variety of pp diseases
132 were identified, and the cows were defined as ill if such an illness was recorded once.
133 Metabolic diseases, such as ketosis, were diagnosed if reductions in feed intake and/or milk
134 yield occurred and if the urine tested positive for ketone bodies (“Ketostix strip”, Bayer,
135 Leverkusen, Germany, positive (+ + +)). Displacement of the abomasum was defined when
136 reductions in feed intake and/or milk yield were observed and abdominal percussion and
137 auscultation were positive. Cows with the following symptoms were suspected of having
138 hypocalcemia: precarious motion, cold body surface, ataxia, and downer cow syndrome.
139 Hypocalcemia was diagnosed if those symptoms disappeared after one infusion of 10 g of
140 calcium as 25 % calcium borogluconate. Fatty liver syndrome was suspected if the cows had a

141 body weight > 800 kg at the time of calving and if the cows had reduced feed intake and/or
142 displacement of the abomasum. Then, a liver biopsy was conducted, and a flotation test was
143 performed. If the biopsy floated in 1020 mg / mL of $CuSO_4$ (= 26 % fat in the liver), fatty
144 liver syndrome was defined (Herdt et al., 1983). A mastitis was diagnosed when the milk
145 showed compositional changes, the udder had signs of inflammation, and the veterinarian
146 detected an elevated cell count in the milk using the California Mastitis Test (CMT + + + ,
147 WDT, Garbsen, Germany) according to Sargeant et al., 2001 (> 100,000 cells / mL). The
148 retention of fetal membranes (RMF) was defined if fetal membranes were still evident 12 h
149 after calving. Uterine content and vaginal discharge before or after twenty-one days of calving
150 indicated metritis/endometritis (Sheldon et al., 2008), which were combined as "metritis" for
151 the statistical analyses.
152
153 ***Endocrine Analyses***
154 ***IGF1.*** The total plasma IGF1 concentrations were determined using a commercial IGF1-
155 ELISA kit (Active IGF1 ELISA; Beckman Coulter, CA, USA) with standard operations
156 according to the manual. The optical density was measured (450 nm), and the concentrations
157 were calculated with Magellan software using the cubic spline modus (Magellan 3.11,
158 Dortmund, Germany). The range of measurements was 10 to 450 ng / mL. The analytical
159 sensitivity was 0.03 ng / mL. The intra- and inter-assay CVs were 3.5 and 8.5 %, respectively.
160
161 ***Quantitative Western Blotting for IGFBP2 and IGFBP3.*** To analyze the concentrations of
162 serum IGFBP2 and IGFBP3 via the binding capacities, a quantitative Western ligand blotting
163 analysis of the serum was performed as previously described (Metzger et al., 2011). Briefly,
164 before electrophoresis on a 5 % stacking / 12 % separating SDS-polyacrylamide gel, the serum
165 samples were diluted 1 : 3 with phosphate buffer (pH 7.4), diluted again 1 : 2 with sample buffer
166 [62.5 mM Tris-HCl (pH 6.8), 2 % (w/v) sodium dodecyl sulfate (SDS), and 10 % (w/v) sucrose],

and boiled (5 min). The separated proteins were transferred to a polyvinyl fluoride membrane (Millipore, Schwalbach, Germany). The blots were blocked using 1 % fish gelatin and incubated with [^{125}I] IGF1. Using recombinant human IGFBP2 and IGFBP3 as internal standards on each blot, the IGFBPs were quantified on a Phosphor-Imager Storm (Molecular Dynamics, CA, USA). The intra-assay variances for the IGFBP2 and -3 determinations in bovine serum were less than 10%. The inter-assay variances ranged between 15 - 20 % , which is acceptable for Western blot based technologies. The lower limits of quantification in the bovine serum were 0.2 ng for IGFBP2 and 1.1 ng for IGFBP3.

Nonesterified Fatty Acids. The serum concentrations of NEFAs were measured using a photometric automatic clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France) by using the NEFA - HR2 Olympus AU 400 Kit (mti-diagnostics GmbH, Idstein, Germany) . The intra-assay CV was 6.2 % .

Statistics

The statistical evaluation of the blood data was performed using SPSS (Version 17.0., SPSS Inc., Chicago, IL, USA). All the data (IGF1, IGFBP2 and -3, and NEFA) were tested for normal distributions using the Kolmogorow-Smirnow test, as modified by Lilliefors (1967), and by a visual inspection of the histograms. The BCS and IGF1 concentrations were tested for differences between the three sampling periods using Student's t-test. Health status was tested for significant differences using Fisher's exact test or a chi-square test. A relative risk assessment was performed to evaluate the potential uses of IGF1, IGFBP2 and NEFA as risk markers for pp diseases. A logistic regression model was developed (odds ratio), and a receiver operating characteristics (ROC) curve was constructed to determine the highest possible sensitivity and specificity. The Youden-Index was calculated using the following

192 formula: sensitivity + specificity - 1. A *P*-value of < 0.05 was considered significant, and a *P*-
193 value between 0.05 and ≤ 0.10 was classified as a statistical tendency.

194 **RESULTS**

195

196 **Diseases**

197 A total of 189 (50.1 %) cows developed production diseases pp, whereas 188 cows (49.9 %)
198 remained healthy during the transition from late pregnancy to early lactation. Of the diseased
199 cows, 73 (34 %) had metabolic diseases, and 142 (66 %) had diseases of the reproductive
200 system (Table 3).

201

202 **Body Conditioning Score and Milk Yield**

203 The BCS of the cows was 4.3 ± 0.03 before calving and decreased pp (+ 3 wk: 3.2 ± 0.03;
204 and + 4 wk: 3.1 ± 0.03; $P < 0.01$). The mean BCS remained constant between 3 and 4 weeks
205 pp ($P = 0.13$). Calving occurred at 281 ± 5 days after AI. The mean ap BCS of the healthy
206 cows was comparable to the cows that developed any production disease after calving (4.30 ±
207 0.04 *vs.* 4.22 ± 0.05; $P = 0.21$). In the cows that developed fatty liver disease after calving (n
208 = 5), the BCS was ap higher (4.90 ± 0.10; $P < 0.01$) compared with the healthy cows. The
209 mean milk yield in the previous lactation of the healthy cows was comparable to that in the
210 cows that developed any production disease after calving (11,279.3 ± 131.1 *vs.* 11,145.3 ±
211 145.2 kg; $P = 0.49$). No significant association between the previous milk yield and the
212 occurrence of any pp production disease was found.

213

214 **Insulin-like Growth Factor 1**

215 In total, the IGF1-concentrations of all the cows ap were higher (88.6 ± 1.5 ng / mL; $P < 0.01$)
216 compared with pp + 3 wk (50.4 ± 1.4 ng / mL) and + 4 wk (53.5 ± 1.3 ng / mL). The cows
217 with a greater loss in BCS between ap and + 3 wk (BCS loss > 1, n = 213) compared with the

218 cows with a smaller BCS loss (BCS loss ≤ 1, n = 164) showed comparable IGF1
219 concentrations before calving (90.2 ± 4.8 *vs.* 91.5 ± 4.0 ng / mL, respectively, $P = 0.68$) but
220 had lower IGF1 concentrations at + 3 wk (44.9 ± 1.8 *vs.* 55.7 ± 2.1 ng / mL, respectively, $P <$
221 0.01) and at + 4 wk (48.1 ± 3.5 *vs.* 57.4 ± 3.6 ng / mL, respectively, $P < 0.01$) after parturition.
222
223 **IGF1 as a predictive marker for postpartum ketosis.** The cows that remained healthy after
224 parturition had higher IGF1 concentrations ap (91.5 ± 1.9 ng / mL) compared with the cows
225 that developed production diseases (ap 85.7 ± 2.2 ng / mL) ($P = 0.05$). However, after
226 parturition, the healthy cows had similar IGF1 levels (+ 3 wk, 51.0 ± 1.9 ng/mL; + 4 wk, 53.9
227 ± 1.8 ng / mL) compared to the cows that developed production diseases (+ 3 wk, 49.7 ± 2.2
228 ng / mL; + 4 wk, 52.9 ± 1.9 ng / mL) ($P = 0.64$ and $P = 0.70$). The cows with pp clinical
229 ketosis had lower IGF1 concentrations ap than the cows that remained healthy after
230 parturition ($P < 0.01$). The IGF1 concentrations of the cows with other diseases were
231 comparable to the concentrations in the healthy cows (Figure 1, $P > 0.05$). The ROC-analysis
232 revealed a significant association between IGF1 and ketosis ($P < 0.01$), the sensitivity of the
233 ap IGF1 concentration as a marker for pp ketosis was 0.87, the specificity was 0.43, the
234 positive predictive value (PPV) was 0.91 and the negative predictive value (NPV) was 0.35.
235 The threshold for distinguishing healthy cows from cows with clinical ketosis was 61.7 ng /
236 mL (Youden-Index = 0.31). The maximal Youden - Index was 0.45 and was found at a cutoff
237 = 90.2 ng / mL; with a sensitivity of 0.51, a specificity of 0.83, a PPV of 0.95 and a NPV of
238 0.21, the AUC was 0.7305 (Figure 2). The cows with 10 ng / mL or higher ap IGF1-
239 concentrations had a 37 % reduced risk for developing clinical ketosis within the first 4 weeks
240 after parturition (OR = 0.97).
241
242 **Insulin-like Growth Factor Binding Proteins**

The cows with ketosis and with RFM had significantly lower IGFBP2 concentrations compared with the healthy cows (Figure 3, $P < 0.01$). The ROC analysis revealed a significant association between IGFBP2 and ketosis ($P = 0.01$), a maximal Youden-Index of 0.37 at a cutoff of IGFBP2 = 3.5 ng/µl with a sensitivity of 0.70 and a specificity of 0.67. The PPV was 0.93 and the NPV was 0.22, corresponding to an AUC of 0.6625. The IGFBP3 concentrations ap were comparable between the healthy cows and the cows that developed any production disease (Figure 4).

Nonesterified fatty acids

The NEFA concentrations before calving were comparable between the healthy cows and the cows that developed a production disease pp (Figure 5, $P > 0.05$). The ROC analyses revealed no significant association between ap NEFA and pp clinical ketosis ($P = 0.07$), a maximal Youden-Index of 0.17 at a cutoff of NEFA = 158 µmol/L with a sensitivity of 0.80 and a specificity of 0.37. The PPV was 0.88 and the NPV was 0.22, corresponding to an AUC of 0.5832.

DISCUSSION

This study aimed to determine whether IGF1 and IGFBPs were ap risk biomakers for pp diseases in dairy cows under field conditions. It is well known that the transition from late pregnancy to early lactation is a critical time period with the occurrence of many orchestrated and interacting endocrine adaptations. If these adaptations are inadequate, the dairy cow is susceptible to metabolic and infectious diseases after calving (Chagas et al., 2007; Williams, 2013; Roche et al., 2013; Lean et al., 2013). In a previous study, it appeared that hepatic derived IGF1 might serve as a promising indicative marker for pp ketosis (Piechotta et al.,

2012). In this study, IGF1 was significantly lower between 242 - 262 days after AI in cows that developed a production disease after calving. Detailed studies on the differences between cows selected for either low or high ap IGF1 levels have revealed that NEFA concentrations increased after the IGF1 concentration was already very low (Piechotta et al., 2013, 2014). Elevated GH in catabolic conditions also has a lipolytic potential combined with catecholamines and low insulin levels and may thus be related to increased NEFA values (Rose et al., 2009; Lanna and Bauman, 1999; Houseknecht and Bauman, 1997). Furthermore, decreased IGF1 concentrations increase the GH release because of a reduced negative feedback at the pituitary level (Hannon et al., 1991; Kobayashi et al., 1999; Lucy et al., 2001). Because of this regulation, IGF1 may be more effective as a very early risk indicator for pp ketosis compared with NEFA or BHBA concentrations. The IGF1 concentrations in the present study were significantly lower in the cows that developed clinical pp ketosis compared to the healthy cows or the cows with other production diseases. Additionally, the NEFA concentrations were not significantly different at this early period of sampling (days 262 - 270 after AI); however, the standard deviation of the NEFA concentrations was higher in this group than in the other groups. This result may indicate that the initiation of lipolysis and the increased NEFA values were a result of lower IGF1 levels; therefore, IGF1 serum levels may be useful for the identification of cows at risk for ketosis as early as possible. A low BCS after calving was associated with a higher risk for ketosis during early lactation (Koeck et al., 2014). In the present study, the ante partum BCS was comparable between the healthy cows and the cows that developed ketosis. Notably, the BCS of the cows in the present study was high, which could be a confounding variable.

The measured IGF1 was defined as the total IGF1, which included all IGF1 molecules free or bound to their IGFBPs. In the circulation, the main binding protein is IGFBP3, which complexes IGF1 with the acid labile subunit and primarily increases the half-life (Rajaram et

295 al., 1997; Nedbal et al., 2000). Notably, the IGFBP3 concentration did not correlate with total
296 IGF1 and was comparable between the healthy cows and the cows with pp diseases. It must
297 be noted that the IGFBPs were determined using a Western blot with a high intra-assay
298 CV % , which might be responsible for this not significant correlation. Piechotta et al. (2013)
299 found that IGFBP3 concentrations decreased towards calving and that cows with low IGF1
300 concentrations did not have significantly lower IGFBP3 serum concentrations in late
301 pregnancy. Feed restrictions can cause lower IGF1 and IGFBP3 concentrations pp, as was
302 demonstrated by Gross et al. (2011), but in other studies, no significant differences between
303 IGF1 and IGFBP3 concentrations with respect to feed restrictions were detected (Laeger et al.,
304 2014). With this respect, factors other than feed intake may regulate hepatic IGF1 production
305 and IGFBP3 concentrations in the blood. One possibility for the absence of a correlation
306 between total IGF1 and IGFBP3 could be that proteases caused a cleavage of IGFBP3 in late
307 pregnancy, as has been described in rats (Wu et al.1999; Fowlkes et al., 1994). Even if human
308 recombinant standards were used for the quantification of bovine IGFBPs, a method
309 dependent reason for the difference between IGF1 and IGFBP3 concentrations is not obvious.
310 This assumption is supported by the high sequence homologies present in the terminal protein
311 domains from IGFBPs of different vertebrate species. Piechotta et al. (2013) published RIA
312 data for IGFBP2 in serum from cattle using a validated assay and obtained concentrations
313 ranging between 0.5 to 2.5 µg / mL; these findings were consistent with Cohick et al. (1992)
314 and Renaville et al. (2000). The IGFBP3 values that were obtained by the Western blot
315 technique were also comparable to published data generated by an ELISA specific for bovine
316 IGFBP3 that ranged between 1 - 3 µg / mL (Hennies and Sauerwein, 2003). This comparison
317 shows that the values were similar even when measured by different techniques. This result
318 may additionally underpin the validity of the method used.
319 Notably, in cows with fatty liver disease, the standard deviations of the IGF1, IGFBP3 and
320 NEFA concentrations were high, which may indicate a difference in the severity of hepatic fat

accumulation reflected by peripheral metabolites. A greater fat content in the liver has also previously been shown to be correlated with greater fat mobilization (Hammon et al., 2009), which may substantiate this speculation. A more detailed differentiation of the fatty liver syndrome would have been of interest, but that was not addressed in the present study.

For a screening test of pp ketosis, a high sensitivity is required. The ROC analyses revealed a threshold of 62 ng / mL for IGF1 with a sensitivity of 0.87 and a specificity of 0.43 and a maximal Youden-Index of 0.31 (cutoff = 90 ng / mL; sensitivity of 0.51 and specificity of 0.83). Therefore, depending on the threshold used, either a higher sensitivity or specificity can be chosen for a potential screening test. However, differences among assays and laboratories suggest that IGF1 concentrations in the blood must be evaluated for a specific laboratory and test method. The assay results for the IGF1 tests indicating a sensitivity of 0.51, a specificity of 0.83 and a Youden-Index of 0.31 appear to be superior to those of NEFA in this study in predicting the risk of ketosis after calving, given the Youden-Index of 0.17 with a sensitivity of 0.80 and a specificity of 0.37. It is notable that whereas IGF1 was less sensitive, it was more specific and had a higher total Youden-Index and a higher area under the curve than the NEFA values. Although ap NEFA and pp BHBA concentrations were both associated with the development of clinical disease, pp serum NEFA concentrations were the most associated with the risk of developing clinical ketosis (Lean et al., 1994; Ospina et al., 2010). However, a closer look at sensitivity and specificity clearly showed that the IGF1 concentrations determined in the present study had a higher specificity and sensitivity than did the NEFA concentrations and could be measured earlier prior to calving. A NEFA concentration measured 14 days before calving had a sensitivity of 0.53 and a specificity of 0.61 for pp clinical ketosis, whereas BHBA had a sensitivity of 0.57 and a specificity of 0.8 after calving (Ospina et al., 2010). In a study by Chapinal et al. (2011), NEFA concentrations obtained one week before calving were associated with an increased risk of RFM, metritis and LDA but not

347 with clinical ketosis (Chapinal et al., 2011). In other studies, the NEFA concentrations 10
348 days before calving indicated a risk for displaced abomasum and BHBA (subclinical ketosis),
349 and in the week after parturition, they indicated a risk for clinical ketosis (LeBlanc, 2010). An
350 extensive evaluation of three different BHBA measuring methods in either milk or urine
351 revealed a high specificity (0.97 and 0.99) for diagnosing ketosis, whereas the sensitivity for
352 the diagnosis was lower (0.55 - 0.98). These data also demonstrate that a sensitivity of 0.9 and
353 a specificity of 0.4 are adequate for predicting ketosis in healthy cows ante partum weeks
354 before clinical symptoms occur. Overall, the data of the present study clearly indicate that
355 IGF1 and IGFBP2 are more accurate and serve as earlier risk indicators for ketosis in cows.
356 Early measurement can lead to appropriate management conditions or feeding additives to
357 assist cows during the transition from late pregnancy to early lactation and to prevent ketosis.
358
359 Notably, the ap IGFBP2 concentrations were lower in the cows with pp clinical ketosis and
360 RFM; again, a high standard deviation in the fatty liver group was obvious. In contrast to the
361 decrease in IGF1 and IGFBP3, the blood concentrations of IGFBP2, the second most
362 abundant binding protein, increased after parturition (Fenwick et al., 2008; Piechotta et al.,
363 2013; Vicini et al., 1991). The IGFBP2 level was shown to be affected by feed restrictions
364 three weeks after parturition in dairy cows (Gross et al., 2011) but not during mid-lactation
365 (Laeger et al., 2014), which indicated that different regulations were operating as calving
366 approached. In a previous study, hepatic IGFBP2 mRNA expression pp was positively
367 correlated with NEFA and BHBA and negatively correlated with hepatic glycogen, blood
368 glucose and IGF1 (Fenwick et al., 2008). However, there appeared to be an early ap signal for
369 IGFBP2 production. In humans, IGFBP2 was shown to affect insulin resistance and played a
370 role in metabolic homeostasis (Ruan and Lai, 2010; Wheatcroft and Kearney, 2009). It is well
371 known that IGFBP2 is suppressed by GH (Hoeflich et al., 2014). Thus, lower IGFBP2 serum
372 levels found in ketosis may be indicative of the increased GH-secretion discussed earlier and

thus nicely support our hypothesis. However, the specific functions of this binding protein regarding metabolic adaptations have not yet been elucidated in cattle. Our data support that monitoring IGFBPs, particularly IGFBP2, might reflect the GH status or even be useful as early biomarkers of distinct health conditions or abnormalities. Supposedly a key metabolic signaling molecule that interacts between metabolism and fertility (Wathes, 2012), this binding protein was indicative of RFM in the present study. Because it has also been shown that cows with higher genetic fertility had a higher hepatic expression of IGFBP2 (Cummins et al., 2012), further studies on the factors affecting this binding protein appear to be promising.

CONCLUSIONS

In conclusion, IGF1 and IGFBP2 levels measured ap between 262 - 270 days after AI were indicative of pp clinical ketosis. Lower IGF1 production in the liver might lead to higher GH secretion, which can initiate lipolysis and result in increasing NEFA concentrations, which may consecutively increase the risk of ketosis. Therefore, compared with NEFA or BHBA, IGF1 and IGFBP2 may represent earlier biomarkers of inadequate metabolic adaptation to the high-energy demands required pp. To use IGF1 concentrations in the blood as a diagnostic test, the threshold must be evaluated for a specific laboratory and test method.

ACKNOWLEDGEMENTS

We thank Martina Baumgarten and Angela Jordan for their technical support with the hormone analyses and Andreas Heinrich, Agrargenossenschaft Uckermark Agrar, Germany, for the kind consent to perform this study on the dairy farm in Göritz.

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Figure Captions

Figure 1. Antepartal (262 - 270 days after AI) insulin-like growth factor 1 (IGF1) concentrations (mean ± SD) of the healthy cows and the cows that developed any postpartal production disease (28 days after calving). * Indicates significant differences ($P < 0.05$) between the healthy cows and the cows with any production disease.

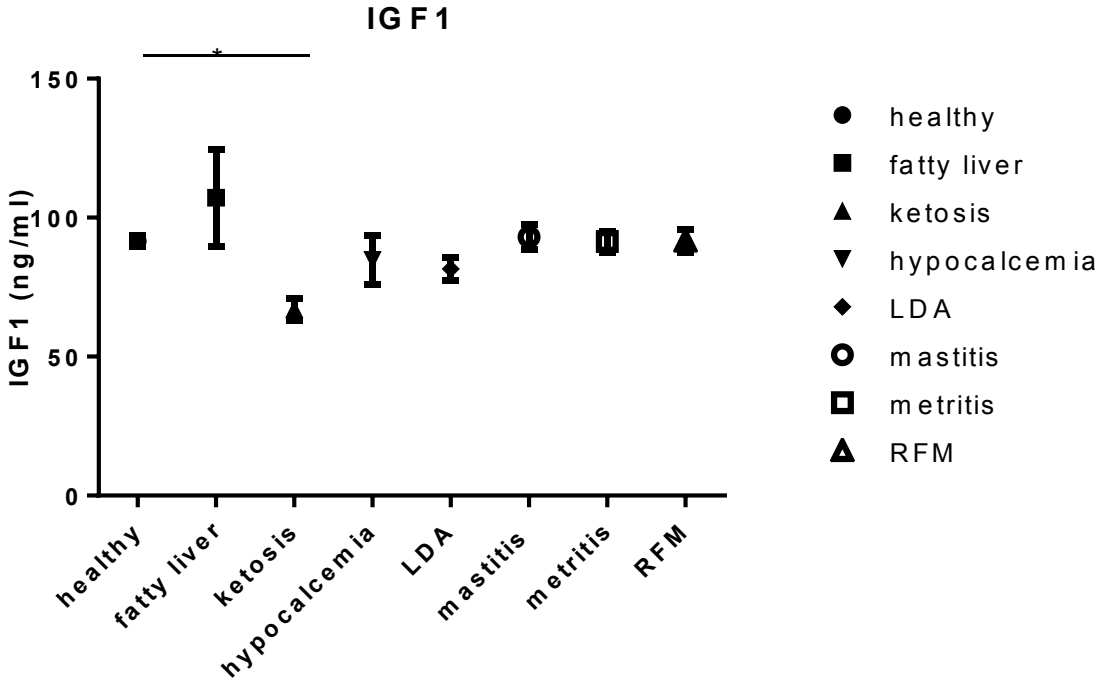
Figure 2. Receiver operating characteristic (ROC) curve for the insulin-like growth factor 1 (IGF1) concentrations in the cows with postpartum ketosis. The two circles indicate the following thresholds: IGF1: 90.2 ng / mL; sensitivity: 0.51; specificity: 0.83; PPV: 0.95; NPV: 0.21; and IGF1: 61.7 ng / mL; sensitivity: 0.87; specificity: 0.43; PPV: 0.91; NPV: 0.35.

Figure 3. Antepartal (262 - 270 days after AI) IGFBP2 concentrations of the healthy cows and the cows that developed a postpartal production disease (28 days after calving). * Indicates significant differences with regard to the healthy cows ($P < 0.05$).

Figure 4. Antepartal (262 - 270 days after AI) IGFBP3 concentrations of the healthy cows and the cows that developed a postpartal production disease (28 days after calving). * Indicates significant differences ($P < 0.05$) between the healthy cows and the cows with any production disease.

Figure 5. Antepartal (262 - 270 days after AI) nonesterified fatty acid (NEFA) concentrations of the healthy cows and the cows that developed a postpartal production disease (28 days after calving). * Indicates significant differences with regard to the healthy cows ($P < 0.05$).

564 **Figure 1**



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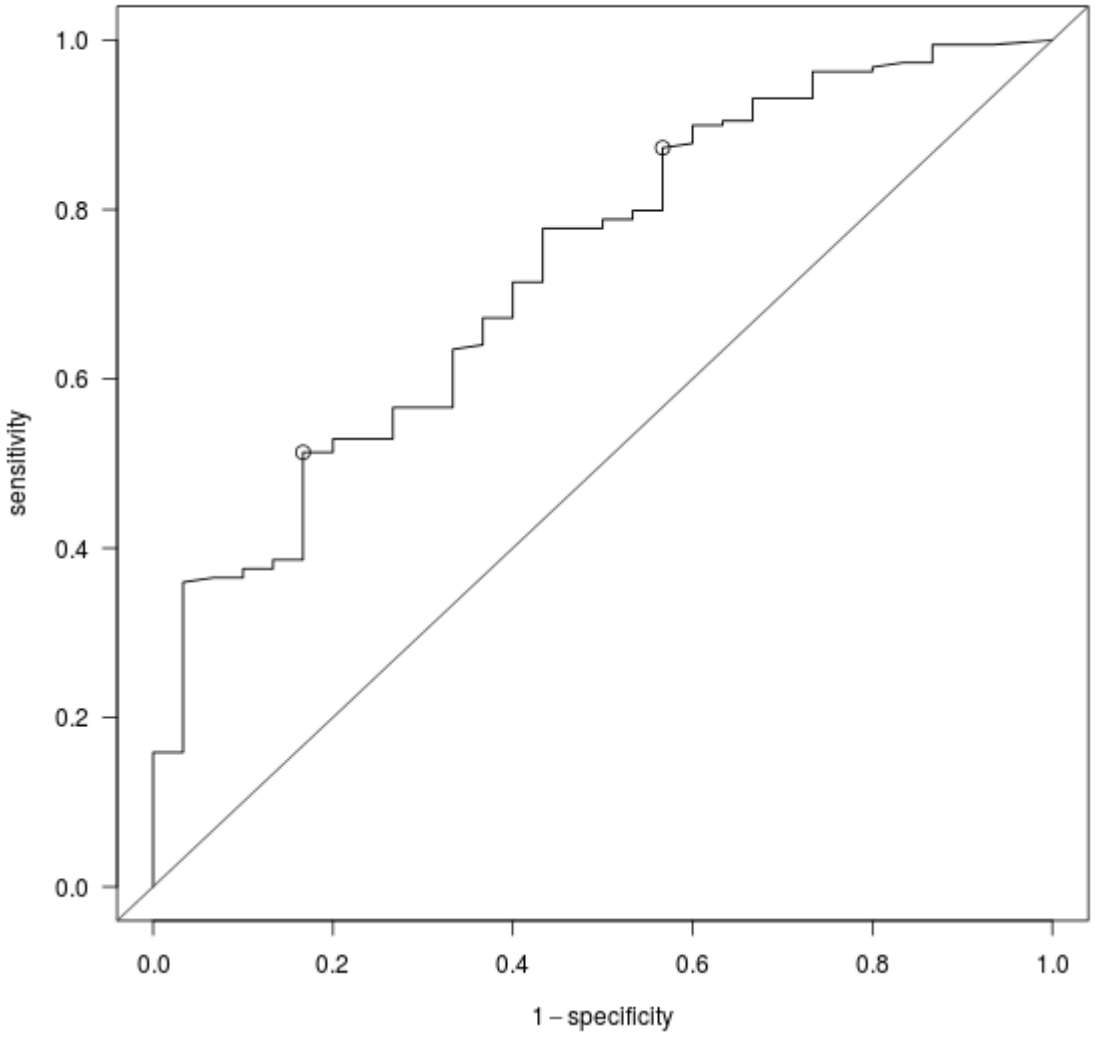
567 **Figure 2**



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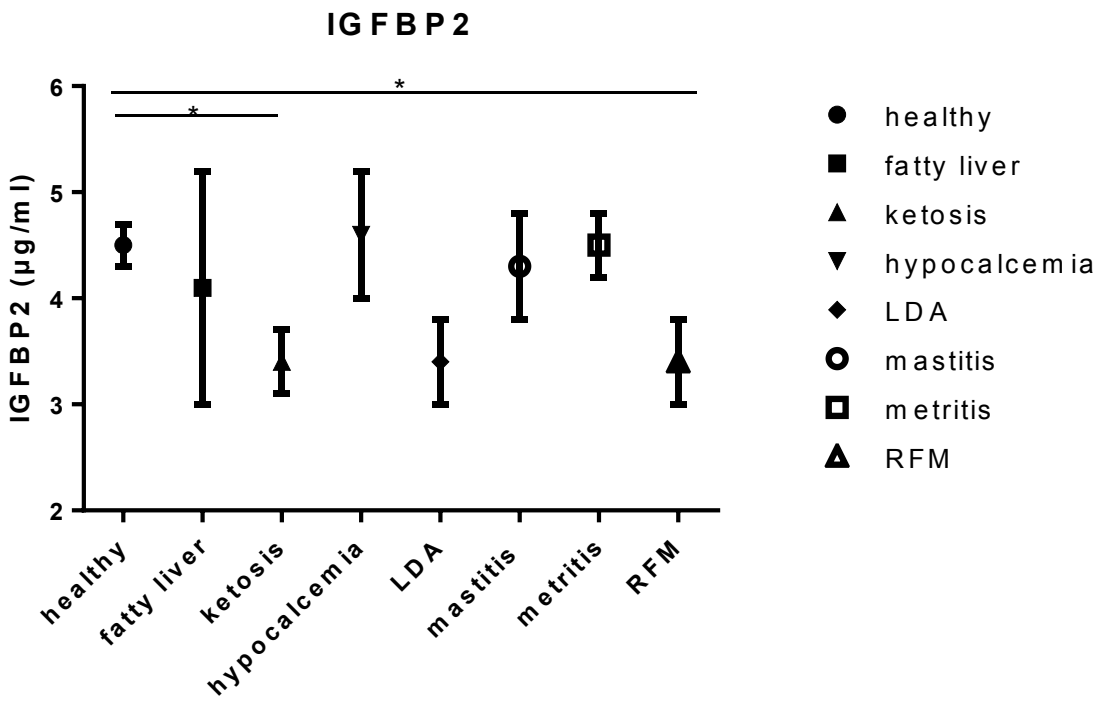
570 **Figure 3**



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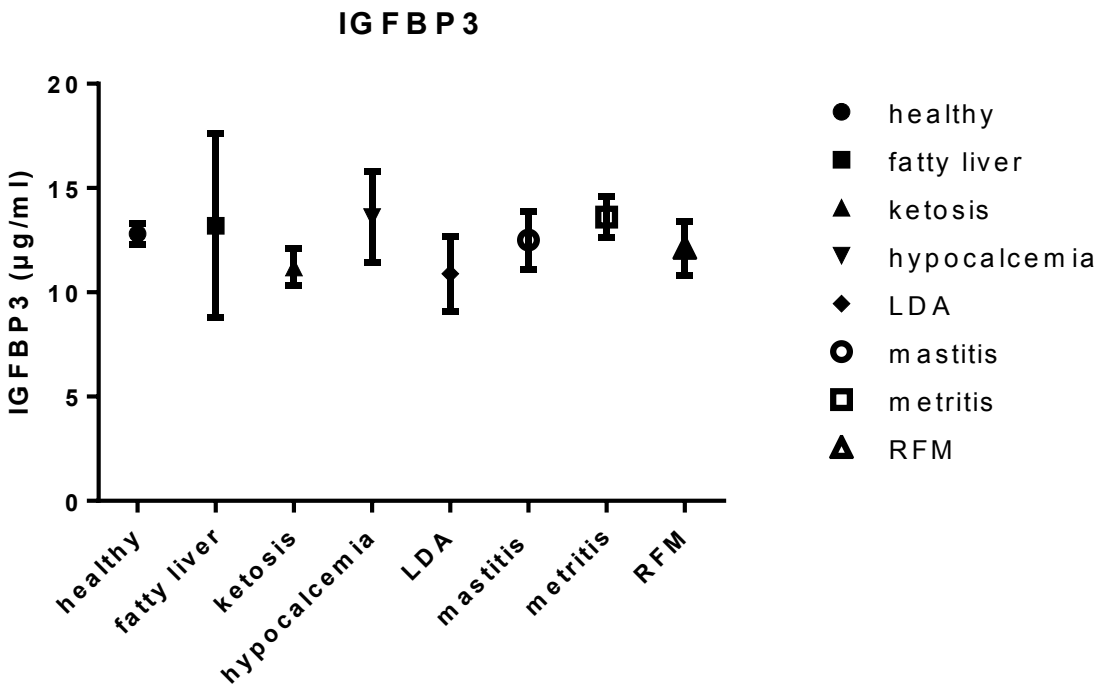
573 **Figure 4**



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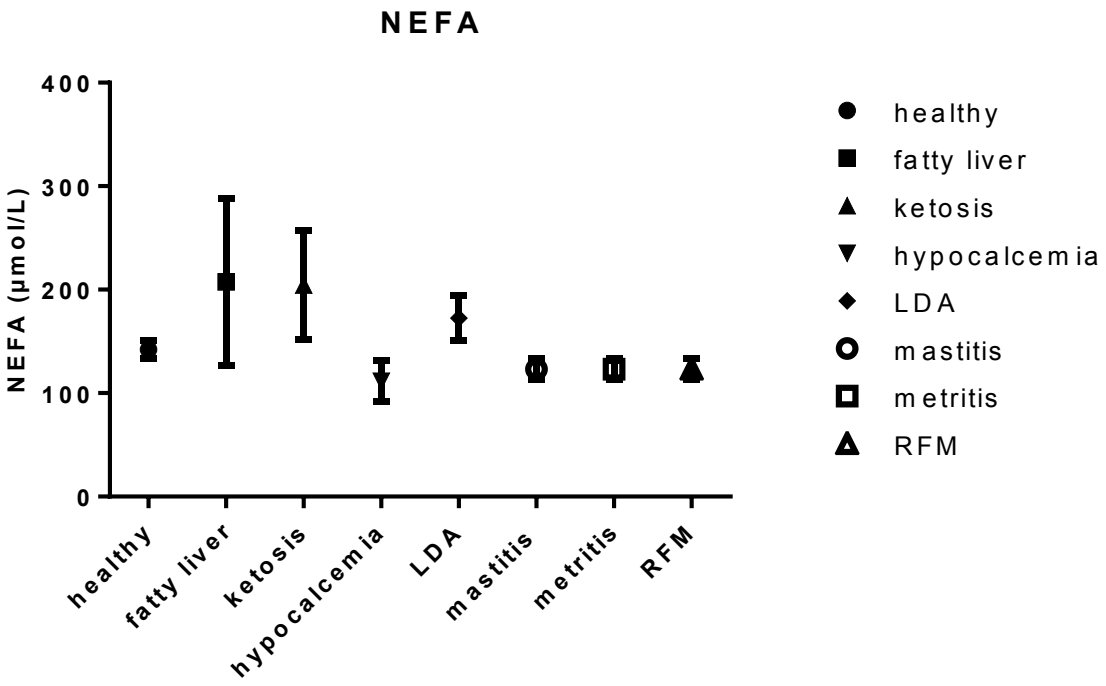
576 **Figure 5**



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580 **Table descriptions**

581 Tabel 1. Ingredients of the total mixed rations fed with regard to the different lactation periods.

582

583 Table 2. Chemical composition of the total mixed rations fed with regard to the different
584 lactation periods

585

586 Table 3. Absolute and relative numbers of cows (percentage of diseased cows out of all tested
587 cows [n = 377]) with any production disease within the first four weeks after calving.

588

589 Table 1.

kg DM	Dry-off ration		Fresh cow ration
	wk 6 - 2 ap	wk 2 - 0 ap	> day 1 pp
Grass silage	10.4	2.4	5.2
Corn silage	1.3	6.6	8.1
Sugar beet pulp	-	-	3.6
Rye straw	0.3	-	-
Wheat	0.4	-	0.6
Straw	2.4	-	0.5
Glycerin[1]	-	-	0.2
Corn	-	-	2.3
Urea[2]	-	-	0.03
Feed-fat[2]	1.7	-	0.4
Soy pellet[3]	-	-	1.7
Rape expellers[3]	-	-	1.2
Cattle salt[4]	0.03	0.03	0.03
Rumen protected protein[1]	-	-	1.9
Propylene-glycol[1]	0.2	-	0.2
Sugar beet chips	0.32	-	-
Flavorful acid salt	0.5	-	-

590 [1]Dr. Pieper Technologie- u. Produktentwicklung GmbH, Wuthenow, Germany, [2] Spezialfutter
591 Neuruppin, Germany, [3]Hauptgenossenschaft Nord AG, Kiel, Germany, [4]Blattin
592 Mineralfutterwerk Seitschen GmbH &Co KG, Göda, Germany, ap = ante partum, pp =
593 postpartum.
594
595
596 Table 2.

	Dry-off ration		Fresh cow ration
	wk 6 - 2 ap	wk 2 - 0 ap	> day 1 pp
NE_L (MJ / kg DM)	5.53	6.39	7.54
Crude ash (g / kg DM)	93	63	53
Crude fat (g / kg DM)	36	29	52
CP (g / kg DM)	134	142	170
Crude fiber (g / kg DM)	267	193	157

597
598 Table 3.
599

Disease	Ketosis	DLA	Rumen atony	Hypo-calcemia	Fatty liver	Metritis	Mastitis	RFM
Number	30	20	14	12	5	66	34	19
Percentage %	7.9	5.3	3.7	3.2	1.3	17.5	9.0	5.0

600 DLA = displacement of the left abomasum, RFM = retained fetal membranes